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(54) Title: PROCESS TO DETECT TOXIC SUBSTANCES IN THE ENVIRONMENT

(57) Abstract

The process according to the invention includes the stages consisting in: placing in an environment to be biomonitored, like air, water, ground, food, clothes, etc., a biosensor consisting in wild type or genetically engineered Dictyostelium cells in conditions apt to development; leaving the cells for 20 hr in the same environment; evaluate the change in morphology of the cell aggregates or the expression of a reporter gene during development. The biosensor for carrying out the process consists of Dictyostelium cells of wild type or transformed with a plasmid containing the gene for a reporter protein. The gene is fused to control elements specific for different Dictyostelium genes, so that the reporter gene is expressed and the reporter protein is synthesised in strains transformed with different plasmids at different times of development, only when the specific regulatory sequences each plasmid contains are activated. The presence of the reporter protein can be detected by naked eye.

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PROCESS TO DETECT TOXIC SUBSTANCES IN THE ENVIRONMENT

Background of the Invention

The present invention relates to a process to detect toxic substances in the environment with the use of a biosensor. The invention concerns also a biosensor for carrying out the process.

The biomonitoring of environmental toxicity is gradually supplanting the chemical monitoring; the advantage of the first one on the latter is that the determination of toxicity with living organisms is much more significant for the influences on the ecosystem and on human health. Furthermore the chemical monitoring allows the determination of the concentration of single substances which are already known to be present in a given environment, but, contrary to the biomonitoring, it does not allow the evaluation of the global toxicity of a given environment, when its nature is unknown.

The living organisms presently utilized for the biomonitoring of water are some species of fishes and macrocrustacea; those employed for the biomonitoring of air are some species of plants and lichens.

The limitations of the employment of these organisms are many. First of all the species of fishes, macrocrustacea, plants and lichens more sensitive to toxic substances are not present in the most polluted environments, as in the vicinity of industry or sewer wastes, or in the cities, where the monitoring necessity is particularly relevant. Transfering fishes or crustacea to a laboratory and mantaining them in water withdrawn elsewhere require complex equipment and specialized personel. Transfering of plants and lichens is pratically impossible since their unrooting generally determines an acute sofference, if not their death. Besides, it is not possible to mantain in a laboratory an air environment identical to the one existing in the center of a city or in a forest. In second place, the biological phenomenon more easily detectable as a consequence of the toxicity of the environment where

an organism lives is its death, which usually requires its exposure for long time to high concentrations of toxic substances. There are biological phenomena more sensitive to the environmental toxicity, but they are generally difficult to be detected and not quantifiable.

Many attempts have been made to develop biosensors capable of bypassing the mentioned limitations. Some pharmaceutical companies sell kits which allow the rapid determination of the toxicity of water samples or of particles collected by filtering air samples. One of the most largely used kit is the one known with the commercial name of "Microtox", which is sold by Beckman. The kit allows to exploit the natural luminescence of a bacterium. If the bacterium looses its luminescence in contact with the kit and a sample of water, the water is toxic.

However this system is based on the synthesis by the bacterium of a single enzyme (luciferase). The biological target is therefore extremely narrow. Indeed this and other similar commercial kits have revealed themselves easy to be employed, but not more sensible of the organisms traditionally employed as biosensors.

Summary of the Invention.

One of the aims of the present invention is to provide a process to detect the thoxic substances in the environment by using a biosensor whose sensibility is significantly greater than the one of the biosensors now available, and which can be used to quantify the extent of toxicity of any type of environment and to detect the presence and determine the nature of specific classes of toxic substances, even if they are present in very low amount, in the air, in the water, in the ground in nature or in a city, and in food and in any material man generally may be in conctact with.

Another aim of the invention is to provide a biosensor for carrying out this process, which allows these results to be obtained at low cost, without complex equipment and specilized personel.

These and other aims and advantages are obtained according to the present invention with the process described in claim 1 and the bio-

sensor used for its carrying out described in claims 3 and 4.

Other advantegeous characteristics are indicated in claims 2, 5, 6 and 7.

The process to detect toxic substances in the environment using a biosensor, according to the invention, includes the stages consisting in :

- placing in an environment to be biomonitored, such as air, ground, water, food, clothes... a biosensor consisting of cells of a wild-type strain or of a genetically enegeneered strain of Dictyostelium discoideum, in coditions apt to development;
- leaving them for 20 hr in the environment; evalueting the morphology of the cell aggregates and/or the expression of one or several reporter genes.

The process, according to the invention, for the preparation of genetically engeneered strains of Dictyostelium discoideum includes the stages consisting in:

- cloning nucleotide sequences in a vector in such a way that a reporter gene is under the control of selected regulatory elements;
- transforming Dictyostelium cells with these vectors.

According to the invention, wild typed or genetically engeneered strains of Dictyostelium are used for the detection of toxic substances in the environment. The survey will simply require the exposure for a few hours in the environment to be monitored of wild type cells of Dictyostelium, in which case the morphological changes of cell aggregates will be followed, or of Dictyostelium cells in which a plasmid containing the gene of a reporter protein has been inserted; in the latter case the reporter gene is fused to regulatory elements derived from different genes of Dictyostelium.

Thus the reporter gene is expressed in different strains only when the selected specific regulatory sequences of Dictyostelium are activated.

The reporter protein will therefore be synthesised only when the regulatory sequences are activated. The presence of the reporter protein in the organism will be detected by nuke eye.

Description of the preferred Embodiment.

In the preferred embodiment, the reporter protein is the enzyme beta-galactosidase of E.coli, which breaks a compound known as X-gal turning it from colourless to blue. Since the organism at the end of development is about 3 mm big and is pratically colourless in nature, but it becomes blue if contains beta-galactosidase, the presence or abscence of the reporter enzyme can be recognized by nuke eye. Different cells of Dictyostelium have been transformed by the inventors with plamsids constructed in such a way that they express or not beta-galactosidase according to whether different amounts of toxic or mutagenic substances are in contact with the cells.

The presence of these substances in the environment may therefore be detected and their concentration evaluated by nuke eye.

Dictyostelium is a lower fungus (a mixomicete) consisting of ameboid cells which in nature feed on bacteria by phagocitosis. The organism is unicellular when it finds bacteria to feed and duplicate. When bacteria are missing, about 100.000 cells call each other by releasing cAMP, and they aggregate, forming a pseudoplasmodium. They initiate a process of differentiation and morphogenesis during which single cells differentiate in two cell types: 80.000 become spores and 20.000 form the stalk which support the sorocarp. The organism may grow in the laboratory on bacteria or in an axenic medium, and milliard of milliards of cells can be easily be obtained. To induce development, cells are removed from the rich medium, washed in buffer and plated on Millipore filters placed on pads embedded with buffer. The filter is in conctact with the air. In laboratories not familiar with growing Dictyostelium cells, it might be more covenient starting from the spores, which can be kept for 1-2 year in a freezer, and induce their germination. Germinated spores (amoebae) cam be plated directly on a filter.

Development proceeds in a syncronous manner and is completed in 20 hr. The morphologically most relevant part of development, the one in which the expression of most developmental genes occurs, lasts 8 hr,

from the 12th to the 20th hr of starvation. The first cell aggregates appear, under a low magnification microscope, 6 hr after starvation (loose aggregates) (see Fig. 1). At 9 hr these aggregates present a tip (tipped aggregates). At 12 hr the tip aggregate elongates and a thin vertical structure appears (first finger). At 15 hr the tip of the first finger begins to invaginate in the cell mass, and a structure appears called mexican hat. At 18 hr the mass formed by the spores begins to climb along the stalk (early and late culminants). At 20 hr the fruiting body is formed, consisting of a stalk 3 mm long, with a sorocarp of a 2 mm diameter at the top.

Dictyostelium is the only organism whose development can be followed entirely in a laboratory in one day. As far as gene expression is concerned, about 4000 new genes are expressed in the last 8-10 hr of development.

The advantages of the utilization of Dictyostelium as a biosensor are at least four. The first one is that the organism develops even if placed on the sidewalk of a street, and is terefore exposed to the air of a city center, or on the floor of an industrial building, or on any piece of land, but also on the surface of a piece of cheese or a piece of cloth. Development occurs perfectly if the filter is embedded with deionized and bidistilled, and therefore absolutely pure, water: this filter can be used as a control; but other filters can in parallel be embedded with various dilutions of water withdrawn from a lake or a river, from the waste of a depuration plant, from the tap of drinkable water, or with water derived from an industrial waste. The organism may therefore be used as a biosensor for any type of environment. If this is natural, it is possible to distinguish whether the toxic substance comes from the ground, when the filter placed on the ground is embedded of pure water and covered with a plastic cover, or from the air, when the filter is placed in a plastic dish which is left uncovered; whether the toxic substance comes from the water when the filter is embedded with water withdrawn from a lake or a river and is enclosed inside a plastic capsule. This is not possible with plants and with many animals, which are contemporary in contact with the

water, the ground and the air of a given environment.

The second advantage is that higher is the toxicity, and at a more precocious stage the development is arrested, so that the morphology of the organism appears different. Therefore anybody, even a person not specifically trained, using a stereoscopic microscope which may cost 3 milions lire, can determine the degree of toxicity of the monitored environment comparing the morphology of the organism with pictures appositely supplied. Even more convenient is to count the fruiting bodies formed after 20 hr of development on the control filter and on the filters exposed to toxic substances. The dilution of the toxic substance(s) which allows the formation of only 50% of fruiting bodies compared to the control filter can be assumed as the EC50 of the toxic substance(s). The determination of the degree of toxicity is thus elementary and can be done at a low cost.

The third advantage is the extreme sensitivity of the system, at least 50 fold higher than the one of the biosensors now available.

The inventors have observed that the development of Dictyostelium is arrested by concentrations of heavy metals (cuprum, zinc, iron, lead, cadmium, mercury, etc) and of organic substances such as CCl_4, chloroform, dichlorometane, trichloroethane, trichloracetic acid, 2,4-dichloro phenoxy acetic acid, aniline, phenol, benzene, toluene, gasolium etc., 20-100 fold lower than those detectable with the other biosensors. It is arrested almost at the same stage by the water withdrawn from Sangone river before it enters in the depuration plant and after it exits, which indicates that with this system a still high degree of toxicity can be detected in depurated water, though with the bisosensors presently used the efficiency of the depuration process appears satisfactory. Twenty samples of real water from industrial wastes in Torino area, from the Po and Dora rivers and even surface waters have all appeared 20-30 fold more toxic than when tested with "Microtox". It has been found slightly toxic the water of the acqueduct of Torino which distributes drinkable water and appear pure with other biosensors. Toxins produced by many fungi have been tested in parallel in mouse and in Dictyostelium: the sensitivity of the latter system is at

least 100 fold greater. The high sensitivity of the system is probably due to the fact that the developmental process is highly complex and requires the coordinate expression of 4.000 new genes in 8 hr mainly in a single type of cells (pre-spore cells). More complex is a system, and more responsive is to a minimal perturbation. The possibility of detecting even minimal degrees of toxicity should allow more rapid interventions by the authorities preposed to human health and to the protection of the ecological systems.

The fourth advantage is the specificity of the system. The inventors have observed that different classes of toxic substances arrest the development in different stages and block the expression of different genes. Therefore, by determining which group of genes is inhibited (with techniques that can be used only in specialised laboratories) it is possible to single out the class of toxic substances present in the examined environment, without the use of chemical tests blindly run. This is not possible with any other biosensor available. To be able to determine which class of toxic substance is present even in a minimal amount in an environment by placing in it a tray with a few dozens filters containing differently engeneered strains of Dictyostelium, will allow immediate interventions to eliminates the toxic substance.

Obviously the invention is not limited to the embodiments here described and illustrated, which have to be considered as examples of the use of Dictyostelium as a biosensor. The invention admits many variant applications, as it will be clear to the experts of the fields: the invention is meant to adopt all the variations included in its sphere, as defined by the following claims.

Claims

1.- Process to detect toxic substances in the environment based on the use of a biosensor, characterized in that it includes the stages consisting in:
- placing in an environment to be biomonitorized, such as air, ground, food, clothes, etc. a biosensor consisting of cells of a wild type strain or of genetically engeneered strains of Dictyostelium in conditions apt to development;
- leaving for 20 hr the cells in such an environment;
- evaluating the morphology of the developing cells aggreagates or the expression of reporter genes.

2.- Process according to claim 1, in which Dictyostelium cells are genetically engeneered, characterized in that it includes the stages consisting in:
- cloning a reporter gene under control elements which allow its expression in specific stages of development;
- transforming different Dictyostelium cells with plasmids containing the reporter gene fused to different control elements in order to obtain Dictyostelium strains which synthesises the reporter protein only in specific stages of development.

3.- Biosensor for carrying out the process according to claim 1, characterized in that it consists in wild type Dictyostelium cells.

4.- Bisoensor for carrying out the process according to claim 1 or 2, characterized in that it consists of Dictyostelium cells in which a plasmid has been inserted containing the gene for a reporter protein; the gene is fused to controlling elements derived from different Dictyostelium genes, so the reporter gene is expressed in different Dictyostelium strains only when the specific controlling elements are activated, and therefore the reporter protein is synthesised only when the same contolling elements are activated; the presence of the repor-

ter protein in the cells can be detected by nuke eye.

5.- Biosensor according to claim 4, characterized in that the reporter protein is E.coli beta-galactosidase.

6.- Biosensor according to claim 4, characterized in that it consists of different Dictyostelium cells transformed with plasmids constructed in such a way that cells express or not beta-galactosidase depending whether they come in contact with different toxic or mutagenic substances; the presence of the reporter protein can be detected by nuke eye.

7.- Biosensor according to claim 4, employed both to detect the extent of toxicity in the environment, on the basis of the stage at which development is arrested and of the number of fruiting bodies formed after 20 hr, and to obtain indications on the class of toxic substances present in the environment, on the basis of the specific genes whose expression is inhibited.

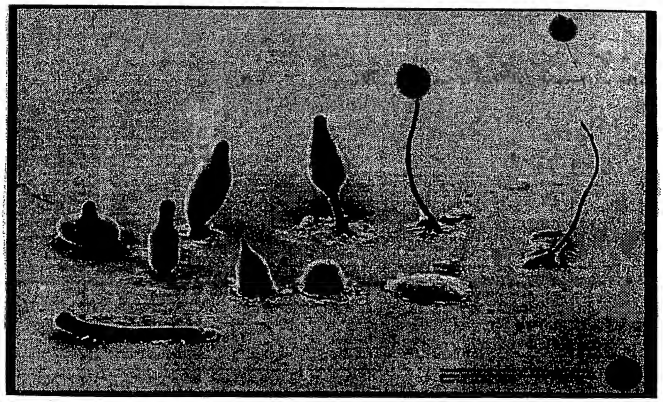

Fig. 1

INTERNATIONAL SEARCH REPORT

Int. tional Application No
PCT/EP 98/05043

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/00 C12Q1/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TILLNER, J. ET AL: "Developmentally regulated promoters from Dictyostelium discoideum as molecular markers for testing potential teratogens." PHARMAZIE, (1996) VOL. 51, NO. 11, PP. 902-906. ISSN: 0031-7144., XP002090801 see page 1 - page 2; figures 4,5 see page 4, column 2 ---	1-7
A	GAYATRI, R. ET AL: "Morphogenesis of Dictyostelium discoideum treated with lindane." BULLETIN OF ENVIRONMENTAL CONTAMINATION AND TOXICOLOGY, (1994) VOL. 52, NO. 6, PP. 871-877. ISSN: 0007-4861., XP002090802 see page 1, column 1; figure 2 see page 6, column 2, line 1 - line 5 ---	1,3

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[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Ir. ational Application No
PCT/EP 98/05043

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	GAYATRI, R. ET AL: "Growth and development of cellular slime mould Dictyostelium discoideum treated with DDT." ENVIRONMENTAL POLLUTION, (1994) VOL. 86, NO. 2, PP. 135-140. ISSN: 0269-7491., XP002090803 see page 1 see page 2, line 5 - line 10; figure 1 see page 6, line 15 - line 27	1,3

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